Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site

GABRIELE WEITZ-SCHMIDT[1], KARL WELZENBACH[1], VOLKER BRINKMANN[1], TETSJI KAMATA[2], JOERG KALLEN[1], CHRISTIAN BRUNS[1], SYLVAIN COTTENS[1], YOSHIKAZU TAKADA[2] & ULRICH HOMMEL[1]

[1]*Novartis Pharma AG, Preclinical Research, Basel, Switzerland*
[2]*The Scripps Research Institute, Department of Vascular Biology, La Jolla, California, USA*
Correspondence should be addressed to G.W.-S.; email: gabriele.weitz@pharma.novartis.com

The β2 integrin leukocyte function antigen-1 (LFA-1) has an important role in the pathophysiology of inflammatory and autoimmune diseases. Here we report that statin compounds commonly used for the treatment of hypercholesterolemia selectively blocked LFA-1–mediated adhesion and costimulation of lymphocytes. This effect was unrelated to the statins' inhibition of 3-hydroxy-3-methylglutaryl coenzyme-A reductase; instead it occurred via binding to a novel allosteric site within LFA-1. Subsequent optimization of the statins for LFA-1 binding resulted in potent, selective and orally active LFA-1 inhibitors that suppress the inflammatory response in a murine model of peritonitis. Targeting of the statin-binding site of LFA-1 could be used to treat diseases such as psoriasis, rheumatoid arthritis, ischemia/reperfusion injury and transplant rejection.

Lymphocyte function-associated antigen-1 (LFA-1, also known as αL-β_2 or CD11a/CD18) is a heterodimeric glycoprotein belonging to the β_2 integrin family[1]. LFA-1 is constitutively expressed on the surface of leukocytes in an inactive state. The integrin requires activation by high concentrations of divalent cations or engagement of the T-cell receptor (TCR) or chemokine receptors to bind to its major counter-receptor, intercellular adhesion molecule-1 (ICAM-1, also known as CD54)[1–3]. LFA-1 is involved in lymphocyte recirculation and leukocyte extravasation to sites of inflammation[1]. The integrin is also important for effective T-cell activation by antigen-presenting cells. Upon ICAM-1 binding, LFA-1 provides a potent costimulatory signal for TCR-activated T cells[4]. Only recently has it been recognized that LFA-1 directly participates in the immunological synapse during TCR ligation by antigen, further underlining the pivotal role of this integrin in immunomodulation[5]. Inhibition of LFA-1 by monoclonal antibodies specific for the antigen has been shown to be effective in animal models of transplantation[6,7], ischemia/reperfusion injury[8], allergic contact dermatitis[9] and arthritis[10]. Clinical studies indicate that use of antibodies against LFA-1 might be an effective therapy for psoriasis[11] as well as for bone-marrow[12] and solid-organ[13] transplantation. However, despite the therapeutic potential of LFA-1 antagonism, as yet no orally active inhibitor of LFA-1 is available for clinical practice.

Using a screening strategy for non-peptidic, small molecule inhibitors of LFA-1, we recently discovered that the natural product lovastatin inhibits LFA-1–mediated adhesion of leukocytes to ICAM-1 and binds to a previously unknown site of the LFA-1 I (inserted)-domain[14], which we have termed 'lovastatin site' (L-site). The crystal structure of the LFA-1 I-domain in complex with lovastatin shows that the L-site is distant from the metal-ion–dependent adhesion site which is critical for ICAM-1 binding. This implies that lovastatin might inhibit LFA-1 via an indirect mechanism[14]. Lovastatin belongs to the drug class of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (that is, statins)[15]. Statins are commonly prescribed to reduce plasma cholesterol levels and consequently the risk of cardiovascular disease[15]. However, there is increasing evidence for additional benefits of statins that cannot be fully explained by their lipid-lowering effect[16,17]. In particular, clinical studies involving transplant recipients indicate possible immunosuppressive actions of statins[17–19].

Here we report a new property of statins entirely unrelated to HMG-CoA reductase inhibition that may explain immunomodulatory effects of these compounds. We show for the first time that besides lovastatin, other statins can block the LFA-1–ICAM-1 interaction by binding to the L-site. We explore the molecular detail of this inhibition and apply this understanding to the design of potent, selective and orally active LFA-1 inhibitors.

Statins inhibit LFA-1–ICAM-1 by binding the LFA-1 L-site

Statins in the β-hydroxy acid form are potent, competitive inhibitors of HMG-CoA reductase, whereas the corresponding lactone form lacks this activity[15]. Both forms, however, and several of their metabolites are found *in vivo* after oral application[20]. To investigate the effect of statins on LFA-1, we used the inactive lactone form (Table 1). Lovastatin, simvastatin, mevastatin and pravastatin were first examined in an ELISA-type LFA-1–ICAM-1 binding assay and a lymphocyte-adhesion assay (Table 1). We also studied the interaction of the compounds with the LFA-1 L-site by nuclear magnetic resonance (NMR) spectroscopy. Simvastatin inhibited LFA-1 binding at concentrations comparable to lovastatin, whereas mevastatin and pravastatin were less active or inactive, respectively (Table 1). These results correlated well with the affinity of the compounds for the L-site. Binding of the compounds to the LFA-1 L-site was confirmed by the chemical shift changes shown by the ^{15}N-HSQC NMR spectra of the isolated LFA-1 I-domain as described[14]. Quantitative analysis of these changes demonstrated that the affinity for the L-site is in the lower micromolar range for all of the statins with the excep-

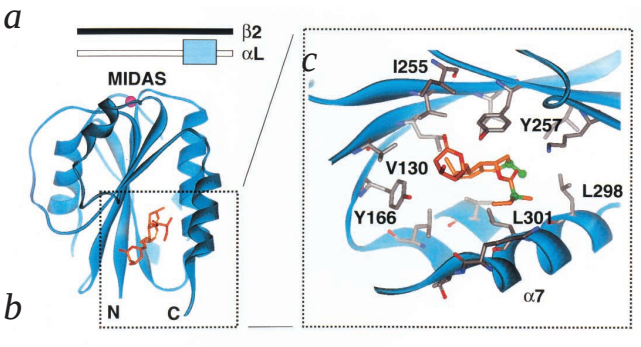

Fig. 1 X-ray structure of the lovastatin–I-domain complex and a close-up of the L-site. ***a***, Schematic representation of the αL-β_2 heterodimeric LFA-1 receptor with the I-domain module (blue box) inserted in the α-chain of the αL-β_2. ***b***, LFA-1 I-domain bound with lovastatin as observed in the X-ray structure. The N- and C-terminal ends are indicated by N and C, respectively; the metal-ion–dependent adhesion site (MIDAS) motif known to be involved in ICAM-1 binding is indicated. ***c***, A close-up of the L-site with some residues in the ligand-binding pocket indicated. Atom positions where the chemical structures of the studied statins differ are indicated by green spheres.

tion of pravastatin, which showed only marginal affinity for the I-domain (Table 1). The observed structure/activity relationship is well explained by the X-ray structure of the I-domain–lovastatin complex (Fig. 1). Mevastatin is less potent than lovastatin due to the missing methyl group at C3, which reduces the number of van der Waals interactions formed between the statin and the L-site (Fig. 1). The lack of activity of pravastatin and its low affinity for the L-site is probably due to its C3 hydroxyl group, which would reside in an unfavorable hydrophobic environment of the L-site (Fig. 1). Simvastatin is as potent as lovastatin suggesting that the extra methyl group at C16 does not compromise L-site binding. This finding is in good agreement with the flexibility of the I-domain α7 helix observed in various X-ray and NMR structures of I-domains solved so far[21–23]. Our data show that lovastatin, simvastatin and mevastatin, but not pravastatin are able to inhibit the LFA-1–ICAM-1 interaction *in vitro* by binding to the L-site of LFA-1. Moreover, the data indicate that compounds of the statin class which bind to the L-site might prevent leukocyte adhesion and extravasation to sites of inflammation and antigen presentation.

Effect of statins on LFA-1– and VLA-4–mediated T-cell costimulation

We next examined the effect of statins on T-cell costimulation specific to LFA-1–ICAM-1 binding. Lovastatin and mevastatin completely blocked the proliferative response of T cells induced by ICAM-1 in combination with a monoclonal antibody against the TCR. However, this inhibition was partially reversed by the addition of mevalonate, the immediate downstream metabolite of HMG-CoA reductase, as shown for lovastatin (Fig. 2*a*). This reversal indicates that the inactive lactone form of lovastatin is hydrolyzed to the active hydroxy acid form, which seems to interfere with the T-cell response by inhibiting HMG-CoA reductase in lymphocytes. It has been reported that HMG-CoA reductase inhibition in lymphocytes leads to impaired proliferative responses to interleukin-2 or phytohemagglutinin[24]. In order to clearly distinguish between effects caused by inhibition of HMG-CoA reductase and LFA-1–mediated effects, we synthesized des-oxo-lovastatin; this statin binds to the L-site and retains its inhibitory effect on LFA-1 function, but cannot be hydrolyzed to the active hydroxy acid form (Table 1). Des-oxo-lovastatin blocked T-cell costimulation induced by the LFA-1–ICAM-1 interaction in a dose-dependent manner (Fig. 2*b*). The presence of mevalonate did not influence this activity, showing that the effect is unrelated to HMG-CoA reductase (Fig. 2*b*). T-cell costimulation mediated by the I-domain–deficient integrin, very late antigen-4 (VLA-4, also known as $\alpha_4\beta_1$ or CD49d/CD29), was not affected by des-oxo-lovastatin at comparable concentrations (Fig. 3*a*). In contrast, the immunosuppressant cyclosporin-A blocked T-cell activation in both assays by interfering with the TCR signal, independent of the mode of costimulation (Fig. 3*b*). Pravastatin did not interfere with T-cell costimulation because

Table 1 Effect of statins, des-oxo-lovastatin and the statin-based LFA-1 inhibitor LFA703 in LFA-1 assays

Assay	Lovastatin	Simvastatin	Mevastatin	Pravastatin	Des-oxo-lovastatin	LFA703
LFA-1–ICAM-1: IC_{50} [μM]	2.1± 0.8 (n = 8)	1.8± 1.3 (n = 5)	8.3± 4.1 (n = 3)	> 100 (n = 5)	1.8 ± 0.5 (n= 5)	0.2 ± 0.1 (n= 5)
HUT78–ICAM-1: IC_{50} [μM]	25.4± 13.3 (n= 5)	22± 4.5 (n = 4)	70.7± 38 (n= 4)	> 100 (n = 3)	26.9 ± 6.2 (n=5)	0.7 ± 0.5 (n= 7)
L-site binding*	+	+	+	–	+	+

The ELISA-type LFA-1–ICAM-1 binding assay (LFA-1–ICAM-1) measures the binding of purified immobilized LFA-1 to recombinant biotinylated ICAM-1. The adhesion assay (HUT78–ICAM-1) quantifies the adhesion of the human T-cell lymphoma cell line HUT78 to immobilized ICAM-1. Binding of the compounds to the LFA-1 L-site (L-site binding) was quantified by NMR spectroscopy. Each value represents the mean ± s.d.; in parentheses, number of independent experiments performed; *uniformly ^{15}N-labeled I-domain was titrated with increasing amounts of compound dissolved in DMSO. The change in the ^{15}N-resonance frequency for the peak corresponding to Thr291 was followed and analysed. + and – correspond to a change > 50 Hz and < 5 Hz at a concentration for the I-domain and compound of 100 μM, respectively.

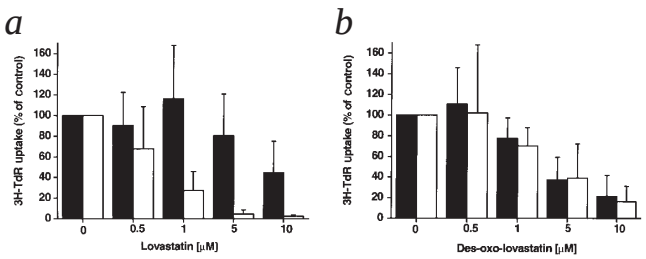

Fig. 2 Effect of lovastatin and des-oxo-lovastatin on LFA-1–specific T-cell costimulation in the presence or absence of mevalonate. ***a*** and ***b***, Effect of lovastatin (*a*) or des-oxo-lovastatin (*b*) on human CD4$^+$ T-cells stimulated by ICAM-1 in combination with an antibody against the TCR. The experiment was performed in the presence (■) or absence (□) of 1 mM mevalonate. Proliferation of T cells was monitored by determining incorporation of [^{3}H]thymidine into the DNA. Bars represent the mean ± s.d. of 2 independent experiments run in triplicate.

this statin does not block LFA-1 as described above, and it does not sufficiently inhibit HMG-CoA reductase in lymphocytes as reported[24] (Fig. 3*c*). A statin-derived compound optimized for LFA-1 binding (LFA703) showed a far more pronounced effect on ICAM-1–induced costimulation than des-oxo-lovastatin, with no effect on VLA-4–induced costimulation up to 1 μm (Fig. 3*d*). This compound is described in more detail below. Taken together, these results show that LFA-1 inhibition by compounds of the statin family is independent of HMG-CoA reductase inhibition. Moreover, they demonstrate that L-site ligands are able to inhibit LFA-1–mediated T-cell costimulation. This inhibition is highly selective for LFA-1 and distinguishes the statin-derived compounds from more broadly acting immunosuppressive drugs like cyclosporin A.

Statins do not block the β_2 integrin Mac-1

Recent studies provide evidence that sites similar to the L-site are also present in other integrins that have an I-domain[25,26]. We therefore investigated the effect of statins on the I-domain–containing β_2 integrin Mac-1 (also known as αM-β_2 or CD11b/CD18)[1]. The tested statins did not inhibit Mac-1 binding to ICAM-1, indicating that the compounds are selective for the L-site of LFA-1 (Fig. 4). This result indicates that the L-site of LFA-1 and putative L-site–like motifs in other I-domains have structural differences that might allow for the design of ligands specific for individual integrins.

Effect of LFA-1 L-site mutations on ICAM-1 binding

The discovery that small molecules such as statins inhibit LFA-1 by binding to a novel site distant from the ICAM-1–binding site strongly suggests an allosteric mechanism of action. The compounds might interfere with the poorly understood process of LFA-1 activation, which is thought to be associated with a conformational change of the I-domain[26]. To investigate this possibility, we substituted L-site residues in close proximity to lovastatin by site-directed mutagenesis. In order to best mimic the effect of a sterically demanding moiety (such as lovastatin) in the vicinity of the L-site, we introduced tryptophan residues in the following positions: V130, Y166, I255 and Y257 (Fig. 1). The site-directed variants were expressed in K562 cells, and LFA-1 expression on each transfectant was analyzed by flow cytometry using IB-4, an antibody against β_2 integrin (Fig. 5). Cells transfected with wild-type LFA-1 did not adhere to immobilized ICAM-1 in the presence of Mg^{++}, but required activation by Mn^{++} for binding to ICAM-1 (Fig. 5). The Y166W, I255W and Y257W variants partially interfered with the LFA-1–ICAM-1 interaction, whereas the V130W mutant completely abrogated adhesion to ICAM-1 despite high-level expression of structurally preserved LFA-1 (Fig. 5). This shows that sterically demanding groups within the L-site impair LFA-1 activation to different degrees. Surprisingly, the replacement of Y257 by alanine resulted in a constitutively active mutant, which, in contrast to wild-type LFA-1, adhered to ICAM-1 in presence of Mg^{++} (Fig. 5). Together, these data demonstrate that the L-site displays hallmarks of an allosteric site naturally present in the dimeric integrin receptor. Moreover, these data provide evidence that L-site ligands like the statins are able to inhibit LFA-1 function via an allosteric mechanism.

Profile of the statin-based LFA-1 antagonist LFA703

In order to design potent LFA-1 inhibitors with reduced activity for HMG-CoA reductase, we used information derived from the mutational analysis and the crystal structure of the LFA-1 I-domain–lovastatin complex. The lovastatin-based LFA-1 inhibitor LFA703 is one example of a series of designed compounds that show increased inhibitory activity for LFA-1, but do not affect HMG-CoA reductase (Table 1). The structure of LFA703 is completely different from earlier non-peptidic, small molecule LFA-1 inhibitors[27,28]. LFA703 binds to the L-site and is 10–36-fold more potent than lovastatin in LFA-1 binding assays (Table 1). The compound blocked LFA-1–induced costimulation of primary T cells in the low nanomolar range, whereas VLA-4–induced costimulation was not inhibited at comparable concentrations (Fig.

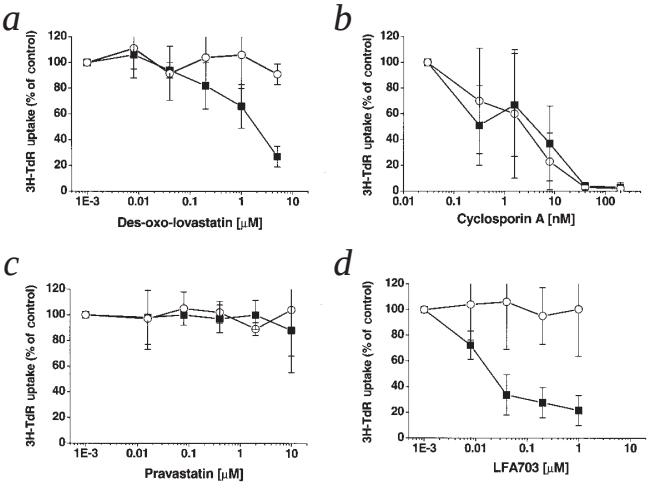

Fig. 3 Effect of des-oxo-lovastatin, cyclosporin A, pravastatin and LFA703 on LFA-1– and VLA-4–induced T-cell costimulation. ***a–d***, Human CD4$^+$ T cells were stimulated by ICAM-1 (■) or VCAM-1 (○) in combination with an antibody against the TCR in the presence of these compounds: des-oxo-lovastatin, an inhibitor of the LFA-1 signal (*a*); cyclosporin A, an inhibitor of the TCR signal (*b*); pravastatin, a negative control (*c*); or LFA703, an inhibitor of the LFA-1 signal (*d*). Proliferation of T cells was monitored by determining incorporation of [^{3}H]thymidine into the DNA. Values represent the mean ± s.d. of 3 independent experiments run in triplicate.

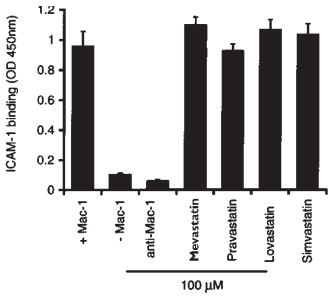

Fig. 4 Binding of ICAM-1 to immobilized Mac-1 in the presence of statins and an antibody against Mac-1. The binding of biotinylated ICAM-1 was quantified in the presence of a blocking antibody against human Mac-1 (10 µg/ml) or the indicated statins (100 µM). Wells containing Mac-1 without addition of inhibitors (+ Mac-1) or not containing Mac-1 (– Mac-1) were used as controls. Bars represent the mean ± s.d. of triplicates. A representative of 3 independent experiments is shown.

3*d*). As shown for the statins, LFA703 had much greater selectivity for LFA-1 than for the integrin Mac-1 (IC_{50} > 100 µM). The compound did not inhibit HMG-CoA reductase up to a concentration of 100 µM (data not shown). The *in vitro* profile of LFA703 is consistent with the low affinity of the V130W mutant for ICAM-1 because the naphthyl moiety of LFA703 would be near the indol moiety of the V130W variant according to the structural model of the lovastatin–I-domain complex (Fig. 1). This indicates that inhibition of LFA-1 by external ligands (such as LFA703) or internal modifications (for example, replacement of valine by tryptophan at position 130) might follow the same mechanism.

Effect of LFA703 in a murine peritonitis model

To determine the *in vivo* activity, we analyzed LFA703 in a murine model of acute inflammation. The model measures thioglycollate-induced migration of neutrophils into the peritoneal cavity of mice. This neutrophil influx can be suppressed by treatment with an antibody against LFA-1. The antibody induced a marked, dose-dependent inhibition with an ED_{50} of 0.6 mg/kg (E_{max}: 68%), demonstrating that the thioglycollate-induced cell migration is dependent on the LFA-1–ICAM-1 interaction (Fig. 6*a*). Oral administration of the small-molecule LFA-1 antagonist LFA703 almost completely inhibited peritonitis with an ED_{50} of 0.4 µg/kg (E_{max}: 81%) (Fig. 6*b*). The high potency and efficacy of LFA703 indicates that L-site ligands of the statin class very efficiently modulate LFA-1 function *in vivo*.

Discussion

HMG-CoA reductase inhibitors are widely used for the treatment of hypercholesterolemia[15]. The clinical benefit of these drugs is commonly thought to result from their ability to reduce cholesterol synthesis by inhibiting the conversion of HMG-CoA to mevalonate[15]. However, HMG-CoA reductase inhibition might affect several other cellular functions since mevalonate is the precursor not only of cholesterol but also of many other nonsteroidal isoprenoid products. Indeed, various *in vitro* and *in vivo* effects of statins are considered to be independent of their cholesterol-lowering effect. Thus, statins inhibit experimental stroke in normal mice but not in mice deficient in endothelial nitric oxide synthase[29]. They increase osteoblast activity and enhance bone formation *in vitro* and in animals[30]. Other reports provide evidence for anti-inflammatory and immunomodulatory properties of statins in transplant recipients and patients with and without coronary heart disease[16,17]. These clinical observations are supported by *in vitro* findings showing that statins suppress T-cell responses[24], reduce expression of class II major histocompatibility complexes on antigen-presenting cells[31] and reduce chemokine synthesis in peripheral blood mononuclear cells[32]. However, all these effects can be reversed by the addition of mevalonate, indicating that they are causally linked to the inhibition of HMG-CoA reductase[24,31,32].

Here we report a property of statins that is unrelated to HMG-CoA reductase inhibition and may at the same time explain their anti-inflammatory and immunomodulatory effects. We show that several statins block the LFA-1–ICAM-1 interaction. This inhibition correlates with the binding of the compounds to a previously unknown site of the LFA-1 I-domain as shown by NMR spectroscopy. We further demonstrate that inhibition of LFA-1 by compounds of the statin class results in decreased lymphocyte adhesion to ICAM-1 and impaired T-cell costimulation. This finding indicates that statins might be able to interfere with anti-inflammatory processes and the immune response. The statins inhibit LFA-1 *in vitro* to various degrees except for pravastatin, which is inactive in the LFA-1 assays. Active metabolites might explain this seeming discrepancy between our *in vitro* results and clinical observations[17,19]. Further investigations will be necessary to determine the relative contribution of LFA-1 inhibition to those effects of statins and their metabolites that benefit transplant patients and patients with cardiovascular disease.

To understand the mechanism by which statins inhibit LFA-1 in more detail, we performed site-directed mutagenesis around the L-site motif. Several mutations led to reduced or completely abolished ICAM-1 binding, indicating that L-site mutations might affect conformational I-domain changes involved in the activation process of LFA-1 (refs. 23,26). We also identified one L-site mutation that constitutively activates LFA-1—probably by keeping the receptor in a conformational state which allows constant ICAM-1 binding. The importance of the L-site for LFA-1 function is further highlighted by the recent observation that binding of ICAM-1 to the metal-ion–dependent adhesion site of the isolated LFA-1 I-domain induces structural changes within the L-site region[33]. Together the results establish the L-site as an

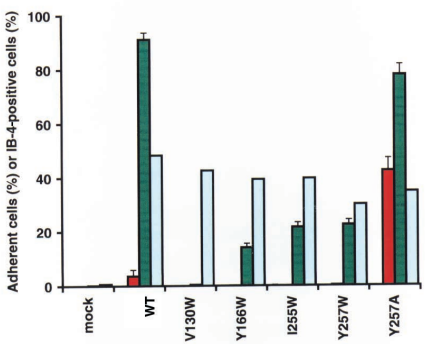

Fig. 5 Effects of L-site mutations on LFA-1–mediated cell adhesion to ICAM-1. Point mutants in the L-site of the αL chain were transiently expressed in K562 cells together with the wild-type β_2 chain. Mock and wild-type (WT) transfected cells were used as controls. Expression of LFA-1 (αL-β_2) was determined by flow cytometry using the antibody IB-4 (▒). Cell adhesion to immobilized ICAM-1 was measured in the presence of Mn^{++} (■) or Mg^{++} (■). Background adhesion was assessed in wells without ICAM-1 (□). Data are expressed as percent of adherent cells or percent-positive cells in flow cytometry. Bars represent the mean ± s.d. of triplicates. A representative of 3 independent experiments is shown.

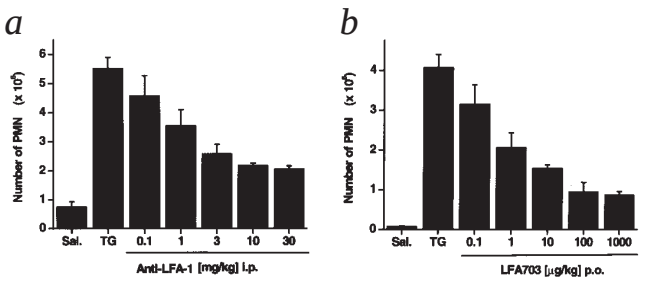

Fig. 6 Inhibition of neutrophil migration in the murine peritonitis model by LFA-1 antagonists. ***a*** and ***b***, Peritonitis was induced in mice by the intraperitoneal (i.p.) injection of thioglycollate (TG). TG-injected mice were treated either with antibody against LFA-1 i.p. (*a*) or with LFA703 orally (p.o.) (***b***). TG or saline (Sal.)-injected mice treated with vehicle alone served as control groups. PMN, polymorphonuclear leukocytes. Bars represent the mean ± s.e.m. of triplicates. A representative of 3 independent experiments is shown. *, $P < 0.001$ (ANOVA).

allosteric site within the intact LFA-1 receptor which allows sensitive regulation of LFA-1–mediated ICAM-1 binding.

Based on the crystal structure of the LFA-1 I-domain–lovastatin complex and information from the mutational analysis of the LFA-1 L-site, we were able to design potent, selective small-molecule inhibitors of LFA-1. The LFA-1 inhibitors described here are the first examples with delineated molecular mechanisms. The compounds do not interfere with the function of other integrins including Mac-1 and VLA-4, indicating high selectivity for LFA-1. Moreover, the remarkable potency of LFA703 in an animal model of inflammation after oral administration indicates that statin-based LFA-1 inhibitors might represent an alternative to antibody blockade.

There is increasing evidence that allosteric sites similar to the LFA-1 L-site are common to I-domain integrins[25,26]. Several of these I-domain integrins have a crucial role in acute and chronic disease states[34]. For example, the β_1 integrins VLA-1 (also known as $\alpha_1\beta_1$ or CD49a/CD29) and VLA-2 (also known as $\alpha_2\beta_1$ or CD49b/CD29) have been recently shown to be involved in delayed-type hypersensitivity reactions and arthritis[35]. The high selectivity of the statins and their derivatives for the LFA-1 L-site indicates that differential targeting of putative L-site–like motifs of other I-domain integrins might be possible and may permit selective intervention in a wide range of pathologies.

In summary, targeting the allosteric L-site of LFA-1 by compounds structurally derived from HMG-CoA reductase inhibitors opens the perspective of developing selective, orally active LFA-1 inhibitors. We anticipate these compounds to have potential applications in autoimmune diseases such as psoriasis and rheumatoid arthritis, ischemia/reperfusion injury and transplantation indications.

Methods

Cell-based LFA-1 adhesion assay. LFA-1–dependent binding of HUT78 cells to immobilized ICAM-1 mouse Cκ fusion protein (ICAM-1) was quantified as described[14]. Briefly, microtiter plates were coated with goat monoclonal antibody against mouse Cκ (1 μg/ml; Southern Biotechnology, Birmingham, Alabama) followed by a blocking step and the addition of ICAM-1 (100 ng/ml). Fluorescently labeled HUT78 cells (1×10^5 cells/well) in Tris-buffered saline containing 1.5% BSA, 5 mM glucose, 2 mM $MgCl_2$ and 2 mM $MnCl_2$ were transferred to the plates and incubated for 0.5 h at 37 °C. The plates were then gently washed and bound cells were quantified by measuring fluorescence.

T-cell costimulation assays specific for LFA-1 and VLA-4. Human $CD4^+$ T cells were negatively selected from Ficoll-isolated peripheral blood mononuclear cells by magnetic cell sorting. Microtiter plates were coated with a mixture of goat monoclonal antibody against mouse Cκ (5 μg/ml) and goat monoclonal antibody against mouse IgG Fc (10 μg/ml; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) in PBS and blocked with 2% BSA in PBS. Human ICAM-1 (1 μg/ml) or human VCAM-1 mouse Cκ fusion protein (VCAM-1; 1 μg/ml) and monoclonal antibody against TCR WT31 (0.01 μg/ml) (Beckton Dickinson Biosciences, Heidelberg, Germany) in PBS containing 2% BSA were added and incubated overnight at 4 °C. Plates were then washed, and 1×10^5 $CD4^+$ cells were transferred to each well and incubated at 37 °C for 4 d in the presence or absence of indicated compounds. In some experiments 1 mM mevalonate was added simultaneously with the compounds. To determine T-cell proliferation, cells were pulsed with [^{3}H]thymidine (1 μCi/well) during the last 16 h of culture. Incorporated radioactivity was quantified using a liquid scintillation counter. Exponential growth of the cells was detected between days 3 and 6 of culture. In the presence of antibody against TCR alone, minimal proliferative responses were observed (< 500 c.p.m.). Costimulation of cells resulted in more than 30000 c.p.m.

LFA-1–ICAM-1 ELISA-type binding assay. The LFA-1–ICAM-1 binding assay was performed as described[14]. Briefly, microtiter plates were coated with purified human LFA-1 and blocked with 1% BSA. Biotinylated ICAM-1 (4 μg/ml) was added in PBS, pH 7.4 containing 2 mM $MgCl_2$ and 1% FCS and incubated for 1 h at 37 °C. Bound ICAM-1 was quantified using streptavidin-peroxidase and 2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (Bio-Rad, Hercules, California) as substrate.

Mac-1–ICAM-1 ELISA-type binding assay. Mac-1 was purified by immunoaffinity chromatography from human polymorphonuclear leukocytes as described[36]. Microtiter plates were coated with purified Mac-1 and blocked with 5% nonfat dry milk. Biotinylated ICAM-1 (2 μg/ml) in Tris buffer, pH 7.4 containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $MnCl_2$ and 0.2% nonfat dry milk was added to the plates and incubated for 1 h at 37 °C. Bound ICAM-1 was quantified using streptavidin-peroxidase and 3,3′,5,5′-tetramethyl-benzidine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as substrate.

NMR binding assay. {^{1}H,^{15}N}-HSQC spectra[37] were recorded on either a 500 MHz or 600 MHz Bruker Avance spectrometer (Bruker AG, Karlsruhe, Germany) at 23 °C. Uniformly ^{15}N-labeled I-domain was titrated with increasing amounts of compound dissolved in DMSO as described[14]. The protein concentration was 100 μM and the maximal ligand concentration varied between 250 μM and 600 μM depending on the ligand. The change in the ^{15}N-resonance frequency for the crosspeak corresponding to the amide group of Thr291 was followed for a qualitative assessment of the binding affinity.

HMG-CoA reductase assay. The radiometric procedure developed by Shum *et al.*[38] was used with slight modifications. Briefly, HMG-CoA reductase (150 μg/ml) was incubated together with compound, 12 μM [^{14}C]HMG-CoA and 200 μM NADPH in 200 μl 0.2 M phosphate buffer (pH 7.2) for 0.5 h at 37 °C. The [^{14}C]mevalonate formed was converted under acidic conditions into [^{14}C]mevalonolactone which was then separated from unreacted substrate by ion-exchange chromatography and quantified by liquid scintillation counting.

Mutational analysis of the LFA-1 L-site. Human αL and β_2 cDNAs were subcloned into pBJ-1 expression vectors, mutated by unique restriction-site elimination[39] and transfected into K562 cells by electroporation. Expression of αL-β_2 was determined by flow cytometry with the antibody IB-4 (Ancell, Bayport, Minnesota). Adhesion of K562 cells expressing αL-β_2 mutants to ICAM-1 was determined as described[40].

Murine thioglycollate-induced peritonitis model. Peritonitis was induced in mice by the intraperitoneal (i.p.) injection of 1 ml 3% thioglycollate (TG)

(Sigma-Aldrich) in saline. Control mice received an injection of sterile saline only. TG-injected mice were treated either with antibody against mouse LFA-1 M17/4.4.11.9 (hybridoma cells from American Type Culture Collection, Rockville, Maryland) i.p. or with LFA703 orally. The control saline or TG-injected group received vehicle i.p. or orally, respectively, at the time of TG injection (vehicle of antibody was saline; vehicle of LFA703 was ethanol/CremophorEL/PBS). Animals were killed 4 h after TG injection and the peritoneal cavity was lavaged with 10 ml of ice-cold Dulbecco's MEM containing 10 U/ml heparin. The volume of lavage fluid was recorded, total cell counts performed and cytospins prepared for differential counting. The cytospin slides were stained with Diff-Quick (Baxter, Healthcare Corp, McGaw Park, Illinois) and the percentage of neutrophils determined.

Acknowledgments

We thank J.P. Evenou for the establishment of the HMG-CoA reductase assay and testing the compounds; L. Michel and C. Wilt for technical assistance; and A.G. Schmidt for critical reading of the manuscript.

RECEIVED 2 FEBRUARY; ACCEPTED 11 APRIL 2001

1. Carlos, T.M. & Harlan, J.M. Leukocyte-Endothelial adhesion molecules. *Blood* **84**, 2068–2101 (1994).
2. Dustin, M.L. & Springer T.A. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* **341**, 619–624 (1989).
3. Campbell, J.J. *et al*. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* **279**, 381–384 (1998).
4. Van Seventer, G.A., Shimizu, Y., Horgan, K.J. & Shaw, S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J. Immunol.* **144**, 4579–4586 (1990).
5. Grakoui, A. *et al*. The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221–227 (1999).
6. Nakakura, E.K. *et al*. Long-term survival of solid organ allografts by brief anti-lymphocyte function-associated antigen-1 monoclonal antibody monotherapy. *Transplantation* **62**, 547–552 (1996).
7. Poston, R.S. *et al.* Effects of humanized monoclonal antibody to rhesus CD11a in rhesus monkey cardiac allograft recipients. *Transplantation* **69**, 2005–2013 (2000).
8. Martin, X. *et al*. Protective effect of an anti-LFA 1 monoclonal antibody (odulimomab) on renal damage due to ischemia and kidney autotransplantation.*Transplant. Proc.* **32**, 481 (2000).
9. Scheynius, A., Camp, R.L. & Pure, E. Unresponsiveness to 2,4-dinitro-1-fluoro-benzene after treatment with monoclonal antibodies to leukocyte function-associated molecule-1 and intercellular adhesion molecule-1 during sensitization. *J. Immunol.* **156**, 1804–1809 (1996).
10. Issekutz, A.C. Adhesion molecules mediating neutrophil migration to arthritis *in vivo* and across endothelium and connective tissue barriers *in vitro*. *Inflamm. Res.* **47**, S123–S132 (1998).
11. Krueger, J. *et al.* Anti-CD11a treatment for psoriasis concurrently increases circulating T-cells and decreases plaque T-cells, consistent with inhibition of cutaneous T-cell trafficking. *J. Invest. Dermatol.* **115**, 333 (2000).
12. Fischer, A. *et al*. Reduction of graft failure by a monoclonal antibody (anti-LFA-1–CD11a) after HLA nonidentical bone marrow transplantation in children with immunodeficiencies, osteopetrosis and Fanconi's anemia: a European Group for Immunodeficiency/European Group for Bone Marrow Transplantation report. *Blood* **77**, 249–456 (1991).
13. Hourmant, M. *et al*. A randomized multicenter trial comparing leukocyte function-associated antigen-1 monoclonal antibody with rabbit antithymocyte globulin as induction treatment in first kidney transplantations. *Transplantation* **62**, 1565–1570 (1996).
14. Kallen, J. *et al.* Structural basis for LFA-1 inhibition upon lovastatin binding to the CD11a I-domain. *J. Mol. Biol.* **292**, 1–9 (1999).
15. Corsini, A., Maggi, F.M. & Catapano, A.L. Pharmacology of competitive inhibitors of HMG-CoA reductase. *Pharmacol. Res*. **31**, 9–27 (1995).
16. Maron, D.J., Fazio, S. & Linton, M.F. Current perspectives on statins. *Circulation* **101**, 207–213 (2000).
17. Katznelson, S. & Kobashigawa, J.A. Dual roles of HMG-CoA reductase inhibitors in solid organ transplantation: Lipid lowering and immunosuppression. *Kidney Int.* **48**, S112–S115 (1995).
18. Wenke, K. *et al*. Simvastatin reduces graft vessel disease and mortality after heart transplantation. *Circulation* **96**, 1398–1402 (1997).
19. Kobashigawa, J.A. *et al.* Effect of pravastatin on outcomes after cardiac transplantation. *N. Engl. J. Med*. **333**, 621–627 (1995).
20. Duggan, D.E.& Vickers, S. Physiological disposition of HMG-CoA-reductase inhibitors. *Drug Metab. Rev.* **22**, 333–362 (1990).
21. Legge, G.B. *et al.* NMR solution structure of the inserted domain of human leukocyte function associated antigen-1. *J. Mol. Biol.* **295**, 1251–1264 (2000).
22. Lee, J.O., Rieu, P., Arnaout, M.A. & Liddington, R. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell* **80**, 631–638 (1995).
23. Qu, A. & Leahy, D.J. Crystal structure of the I-domain from the CD11a/CD18 (LFA-1, αL β2) integrin. *Proc. Natl. Acad. Sci. USA* **92**, 10277–10281 (1995).
24. Kurakata, S., Kada, M., Shimada, Y., Komai, T. & Nomoto, K. Effects of different inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, pravastatin sodium and simvastatin, on sterol synthesis and immunological functions in human lymphocytes *in vitro*. *Immunopharmacol.* **34**, 51–61 (1996).
25. Emsley, J., Knight, C.G., Farndale R.W., Barnes M.J. & Liddington, R.C. Structural basis of collagen recognition by integrin $\alpha_2\beta_1$. *Cell* **100**, 47–56 (2000).
26. Leitinger, B. & Hogg, N. From crystal clear ligand binding to designer I domains. *Nature Struct. Biol.* **7**, 614–616 (2000).
27. Kelly, T.A. *et al*. A small molecule antagonist of LFA-1-mediated cell adhesion. *J. Immunol*. **163**, 5173–5177 (1999).
28. Liu, G. *et al.* Discovery of novel *p*-arylthio cinnamides as antagonists of leukocyte function-associated antigen-1/intracellular adhesion molecule-1 interaction. 1. Identification of an additional binding pocket based on an anilino diaryl sulfide lead. *J. Med. Chem.* **43**, 4025–4040 (2000).
29. Endres, M. *et al*. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **95**, 8880–8885 (1998).
30. Mundy, G. *et al*. Stimulation of bone formation *in vitro* and in rodents by statins. *Science* **286**, 1946–1949 (1999).
31. Kwak, B., Mulhaupt, F., Myit, S. & Mach, F. Statins as a newly recognized type of immunomodulators. *Nature Med.* **6**, 1399–1402 (2000).
32. Romano, M. *et al*. Inhibition of monocyte chemotactic protein-1 synthesis by statins. *Lab. Invest*. **80**, 1095–1100 (2000).
33. Huth, J.R. *et al.* NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen ligand binding. *Proc. Natl. Acad. Sci. USA* **97**, 5231–5236 (2000).
34. Mousa, S.A. &. Cheresh, D.A. Recent advances in cell adhesion molecules and extracellular matrix proteins: potential clinical implications. *Drug Discov. Today* **2**, 187–199 (1997).
35. de Fougerolles, A.R. *et al.* Regulation of inflammation by collagen-binding integrins α1β1 and α2β1 in models of hypersensitivity and arthritis. *J. Clin. Invest*. **105**, 721–729 (2000).
36. Diamond, M.S. *et al.* ICAM-1 (CD54): A counter-receptor for Mac-1 (CD11b/CD18). *J. Cell. Biol.* **111**, 3129–3139 (1990).
37. Palmer III, A.G., Cavanagh, J., Wright, P.E. & Rance, M. Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectrocsopy. *J. Magn. Reson.* **93**, 151–170 (1991).
38. Shum, Y.Y. *et al*. Development, validation, and interlaboratory comparison of an HMG-CoA reductase inhibition assay for quantitation of artovastatin in plasma matrices. *Ther. Drug Monit.* **20**, 41–49 (1998).
39. Deng, W.P.& Nickoloff, J.A. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem*. **200**, 81–88 (1992).
40. Kamata, T., Wright, R. & Takada, Y. Critical threonine and aspartic acid residues within the I domains of beta 2 integrins for interactions with intercellular adhesion molecule 1 (ICAM-1) and C3bi. *J. Biol. Chem*. **270**, 12531–12535 (1995).